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PRINCIPAL INVESTIGATOR: I. Bernard Weinstein, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

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I. Bernard Weinstein, M.D.

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Columbia University
New York, New York 10032

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Our previous studies concentrated on the role of cyclin D1 in breast cancer. In recent studies we have found that cyclin E, which also plays a critical role in the G1 to S progression of the cell cycle, is also frequently overexpressed in breast cancer cell lines. We found that ectopic overexpression of cyclin E stimulates the growth of fibroblast cell lines but inhibits the growth of some mammary epithelial cell lines. The latter effects are associated with increased expression of $p27^{Kip1}$. These findings, together with our previous results, provide evidence for the existence of homeostatic feed-back loops between cyclin D1 and $p27^{Kip1}$ and between cyclin E and $p27^{Kip1}$. This may explain the otherwise paradoxical finding that human breast cancer cell lines and primary tumors often express relatively high levels of $p27^{Kip1}$ even though this protein is an inhibitor of cell cycle progression. We found that overexpression of cyclin E in mammary epithelial cells was associated with increased resistence to growth inhibition by TGF-β. This effect, together with other effects of cyclin E, cyclin D1 and $p27^{Kip1}$, may contribute to mammary tumorigenesis. These abnormalities might be exploited in the chemoprevention and therapy of breast cancer.

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I. Bernard Weinstein 9/1/97

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THE ROLE OF CYCLIN D1 OVEREXPRESSION IN BREAST CANCER PROGRESSION

(5) INTRODUCTION

Nature of the Problem. Although there is considerable evidence that the majority of human breast cancers are caused by environmental factors (including dietary factors) and reproductive factors, rather than hereditary factors, the specific causes of human breast cancer are not known with certainty. Furthermore, although exciting progress has been made in identifying mutations and or aberrant expression of cellular oncogenes and tumor suppressor genes in human breast cancers, the precise mechanisms responsible for the uncontrolled proliferation of breast cancer cells, the apparent genomic instability of malignant breast tumors, and the often relentless course of tumor progression, are poorly understood at the present time.

Background. Cyclins are a recently identified family of proteins that regulate the passage of cells through the G1, S, G2 and M phases of the cell cycle (for review see 1-5). These proteins complex with specific cyclin-dependent serine-and threonine-protein kinases (CDKs), thereby regulating the activity of these kinases. This process is further modulated by the phosphorylation and dephosphorylation of CDK proteins by specific protein kinases and phosphatases, and by specific inhibitory proteins called CDKIs. At least 7 distinct cyclin genes have been identified in the human genome and at least six CDKs (CDK 1-6) form complexes with these cyclins. Based on their conserved sequence motifs with cyclins in other species and their patterns of expression and apparent functional roles during the cell cycle, they are grouped into three categories: G1 cyclins, including cyclins D1-3, and E; the S-phase cyclin, cyclin A; and G2/M phase cyclins, cyclins A, B1 and B2. Cyclin B is the best characterized mammalian cyclin. It complexes with CDK1 (CDC2) to regulate both mitotic entry and exit. It is not known whether cyclin B1 and B2 are interchangeable. After stimulating quiescent cells by growth factors, cyclin D1 is expressed maximally during mid to late G1, although it appears to be expressed at a constant level in continuously dividing cells. Cyclin D1, and cyclins D2 and D3, associate with CDKs 4 and 6 thereby activating their activities. This leads to phosphorylation and inactivation of the tumor suppressor protein Rb, thus causing activation of the transcription factor E2F which enhances S phase progression. It is not known whether all of the biologic effects of cyclin D1 are mediated via the Rb protein. Cyclin E acts in late G1 by activating CDK2 and cyclin A acts in the S and G2/M phases by activating CDK2.

Several lines of evidence implicate the role of cyclins D1 and E in human breast cancer. 1) The cyclin D1 genes originally termed Prad 1, is located at chromosome 11q13. We and other investigators have demonstrated amplification and/or increased expression of cyclin D1 in a subset of primary human breast cancer cell lines (6-9). 2) Increased expression and deregulation in the expression of cyclin E have been described in human breast tumors and cell lines (1, 2, 4, 10, 11). It is of interest that the increased expression of cyclin D1 and cyclin E do not always correlate with amplification of the corresponding genes.

Our laboratory has previously described amplification and/or increased expression of cyclin D1 in human tumors of the esophagus, liver, and colon (3, 4, 12, 13). We have also demonstrated that stable overexpression of cyclin D1 shortens the G1 phase of the cell cycle and

enhances malignant cell transformation (5). These studies from our laboratory, coupled with the other evidence (cited above) of abnormalities in cyclins in human breast cancers, provide the basis of this grant.

Purpose of the present work. The overall purpose of this work is to examine the hypothesis that abormalities in the expression of cyclin D1 and related cell cycle control genes play important roles in multistage breast carcinogenesis by enhancing the process of tumor progression. These studies might provide new biomarkers and diagnostic tools to more precisely detect and stage breast tumors. This approach could also lead to the development of agents that inhibit the action of specific cyclins or cyclin-dependent protein kinases in human tumors, and thus lead to novel strategies for breast cancer chemoprevention and therapy. If abnormalities in the expression of cyclin genes enhance genomic instability, as suggested by our recent studies (14), then such inhibitors might specifically block the progress of tumor progression and the emergence of hormone independent and drug resistant variants of breast tumors.

Methods of approach. As discussed above, the cyclin D1 gene is frequently amplified and/or overexpressed in primary human breast cancers and breast cancer cell lines, but the functional and prognostic significance of this finding is not known. We are using well defined normal human mammary epithelial and human breast cancer cell culture systems to analyze the role of cyclin D1 in cell cycle control, gene expression and amplification, cell transformation and tumorigenicity. Similar studies are also being done with cyclin E. A major strategy employed in our studies is to utilize gene transfer methods to develop derivatives of normal mammary epithelial cells that stably overexpress either cyclin D1 or cyclin E and then examine possible effects on growth control, differentiation and various cell cycle parameters (6, 9, 11, 15).

The studies supported by this grant have employed several experimental systems, including: 1) carcinogen-induced rat mammary tumors; 2) cell lines derived from either normal mammary epithelium or breast carcinomas; 3) genetically engineered derivatives of these cell lines that stably overexpress cyclin D1 or cyclin E; and, 4) a series of primary human breast carcinomas studied by immunohistochemistry.

(6) BODY

(a) Previous studies on this grant carried out in 1994-1996

In our previous annual progress reports we provided the first evidence that there is a marked increase in the expression of cyclin D1 in NMU-induced primary rat mammary tumors (16). Significant but less striking increases in the expression of cyclin E, cyclin A, CDK2 and CDK4 were also seen (16). We also described amplification and increased expression of cyclin D1 in several human breast cancer cell lines (6). We found that stable overexpression of an exogenous cyclin D1 cDNA in the HBL-100 and HC11 mammary epithelial cell lines, inhibited growth and enhanced apoptosis (6, 9). An unexpected finding was that increased expression of

cyclin D1 in HBL-100, HC11 and MCF-10F cells was associated with increased expression of the cell cycle inhibitory protein $p27^{Kip1}$ (9). These findings provide evidence for a homeostatic feedback inhibitor loop between cyclin D1 and $p27^{Kip1}$ (9). In a separate study we found that overexpression of cyclin E, which frequently occurs in human breast cancers, in HC11 cells also inhibited growth and induced expression of $p27^{Kip1}$ (11). During the past year, the latter findings were examined in greater detail, as described below.

(b) Effects of cyclin E overexpression on cell growth and response to transforming growth factor β depend on cell context and $p27^{Kip1}$ expression (15).

Background. Human breast tumors often display increased expression and dysregulation of cyclin E (10, 11), suggesting that this might contribute to their abnormal growth. Cyclin E binds to and activates CDK2 and this complex plays a critical role in cell cycle progression, acting in late G1 after cyclin D1. However, in 1996 we reported that overexpression of a human cyclin E cDNA in the nontransformed mouse mammary epithelial cell line HC11 resulted in increased expression of the cell cycle-inhibitory protein $p27^{Kip1}$ and inhibition of cell growth (11). To further address the significance of this finding and the role of cell context, in the present study we analyzed, in parallel, the effects of cyclin E overexpression in two fibroblast cell lines (Rat1 and NIH3T3) and three nontumorigenic mammary epithelial cell lines (the human mammary cell lines 184B5 and MCF-10F and the mouse mammary cell line HC11).

Experimental Methods. Derivatives of the above described 5 cell lines were obtained by infecting the cells with retrovirus particles containing a cyclin E cDNA sequence and selection for hygromycin resistant cells, as previously described (11). Vector control cells infected with retrovirus particles lacking the cyclin E sequence (11) were also prepared and always analyzed in parallel with the cyclin E overexpressor cells for several phenotypic properties. The details for the procedures used for cell culture, the construction of the retrovirus vectors, viral transduction, growth curves, assays for anchorage-independent growth, flow cytometry for cell cycle kinetics, western blot analyses, and assays for cyclin/CDK kinase activities have been described in our previous publications (6, 9, 11).

RESULTS AND DISCUSSION

To further address the role of cyclin E in mammary tumorigenesis, and the effects of cell context, we analyzed in parallel the effects of cyclin E overexpression in two fibroblast cell lines (Rat1 and NIH3T3) and three nontumorigenic mammary epithelial cell lines (the human mammary cell line 184B5 and MCF-10F and the mouse mammary cell line HC11). We found that increased expression of cyclin E was associated with increased cyclin E-associated kinase activity in Rat1, NIH3T3, and MCF-10F cells but not in HC11 and 184B5 cells. It is of interest that the derivatives of the latter two cell lines showed increased expression of $p27^{Kip1}$ and inhibition of cell growth. There was a shortening of the G_1 phase in the derivatives of the Rat1 and MCF-10F cells but not in the derivatives of the other three cell lines. Contrary to a previous hypothesis,

overexpression of cyclin E was not able to confer anchorage-independent growth in any of these cell lines. However, overexpression of cyclin E was associated with increased resistance to transforming growth factor β-mediated growth inhibition in the 184B5 and HC11 cells and a decrease in transforming growth factor β stimulation of the Rat1 and NIH3T3 fibroblasts.

Thus, overexpression of the same cyclin E cDNA has cell type-specific effects on various growth parameters. We previously provided evidence that in some mammary epithelial and breast cancer cell lines there is a homeostatic feed-back loop between cyclin D1 and $p27^{Kip1}$ (9). The present study provides evidence that there is also a homeostatic feed-back loop between cyclin E and $p27^{Kip1}$, which is also cell context dependent. Our results are not confined to cell culture systems since, although $p27^{Kip1}$ is a putative tumor suppressor, in studies now in progress we have found that $p27^{Kip1}$ is also expressed at relatively high levels in about 50% of primary human breast cancers.

The above study was recently published in the journal Cell Growth and Differentiation (15). A reprint of this paper is enclosed in the Appendix. The reader is referred to this reprint for specific details, and the Tables and Figures that provide the actual data.

(c) <u>Overexpression of Cyclin D1 in Primary Human Breast Cancers and Correlations with Various Clinical and Pathologic Parameters</u>

In view of our previous findings on cyclin D1 overexpression in NMU-induced rat mammary tumors and in human mammary epithelial and breast cancer cell lines, it was of interest to examine cyclin D1 expression in a series of various types of primary human breast cancers and search for possible correlations with various clinical and pathologic parameters. Therefore, we determined the levels of cyclin D1 expression in 140 cases of primary breast cancer by immunostaining of formalin fixed-parrafin embedded tissue, using an immunoperoxidase technique. The immunostaining method, controls for specificity and the scoring procedure were essentially the same as previously employed by our laboratory in a study on cyclin D1 in human colon cancer (13). Staining intensities of 2 or 3 were considered positive for overexpression since 0 or 1 intensities, but never 2 or 3, were seen in normal adjacent tissue. In addition, at least 5% of the tumor cells in a given case had to show overexpression for the case to be considered positive.

The overall positive rate for cyclin D1 overexpression in this group of cases was 68%. We found a significant correlation between cyclin D1 overexpression and the following parameters: estrogen receptor positivity, high tumor grade, aneuploidy, high proliferation index and low expression of HER 2/c-<u>neu</u>. Follow-up information on these cases in terms of recurrence of disease, response to therapy and survival is not yet available. Therefore, the clinical significance of these findings with respect to prognosis remains to be determined. Nevertheless, it is apparent from our studies, and those by other investigators, that overexpression of cyclin D1 is one of the most frequent characteristics of human breast cancer. A manuscript describing these results is in preparation.

(7) CONCLUSIONS

Our previous studies concentrated on the role of cyclin D1 in breast cancer. In recent studies we have found that cyclin E, which also plays a critical role in the G1 to S progression of the cell cycle, is also frequently overexpressed in breast cancer cell lines. We found that ectopic overexpression of cyclin E stimulates the growth of fibroblast cell lines but inhibits the growth of some mammary epithelial cell lines. The latter effects are associated with increased expression of $p27^{Kip1}$. These findings, together with our previous results, provide evidence for the existence of homeostatic feed-back loops between cyclin D1 and $p27^{Kip1}$ and between cyclin E and $p27^{Kip1}$. This may explain the otherwise paradoxical finding that human breast cancer cell lines and primary tumors often express relatively high levels of $p27^{Kip1}$ even though this protein is an inhibitor of cell cycle progression. We found that overexpression of cyclin E in mammary epithelial cells was associated with increased resistence to growth inhibition by TGF-β. This effect, together with other effects of cyclin E, cyclin D1 and $p27^{Kip1}$, may contribute to mammary tumorigenesis. In a separate study of 168 primary human breast cancers we found increased expression of cyclin D1 in 68% of these cases. Therefore, overexpression of cyclin D1 is one of the most frequent molecular defects in human breast cancer. These various abnormalities in cell cycle control proteins might be exploited in the chemoprevention and therapy of breast cancer.

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Effects of Cyclin E Overexpression on Cell Growth and Response to Transforming Growth Factor β Depend on Cell Context and p27^{Kip1} Expression[1]

Alessandro Sgambato,[2] Yuichiro Doki, Ira Schieren, and I. Bernard Weinstein[3]

Columbia-Presbyterian Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Abstract

Human tumors often display increased expression of cyclin E, suggesting that this might contribute to their abnormal growth. However, we reported recently that overexpression of a human cyclin E cDNA in the nontransformed mouse mammary epithelial cell line HC11 resulted in increased expression of the cell cycle-inhibitory protein p27^{Kip1} and inhibition of cell growth. To further address the role of cell context, in the present study we analyzed in parallel the effects of cyclin E overexpression in two fibroblast cell lines (Rat1 and NIH3T3) and three nontumorigenic mammary epithelial cell lines (the human cell lines 184B5 and MCF-10F and the mouse cell line HC11). This was associated with increased cyclin E-associated kinase activity in Rat1, NIH3T3, and MCF-10F cells but not in HC11 and 184B5 cells. The derivatives of the latter two cell lines showed increased expression of the p27^{Kip1} protein and inhibition of cell growth. There was a shortening of the G_1 phase in the derivatives of the Rat1 and MCF-10F cells but not in the derivatives of the other three cell lines. Contrary to a previous hypothesis, overexpression of cyclin E was not able to confer anchorage-independent growth in any of these cell lines. However, overexpression of cyclin E was associated with increased resistance to transforming growth factor β-mediated growth inhibition in the 184B5 and HC11 cells and a decrease in transforming growth factor β stimulation of the Rat1 and NIH3T3 fibroblasts. Thus, overexpression of the same cyclin E cDNA has cell type-specific effects on various growth parameters. Therefore, additional studies are required to better understand the significance of the frequent increase of cyclin E expression in human tumors.

Introduction

Cyclins are a family of genes involved in the regulation of cell cycle progression in eukaryotes through mechanisms that have been highly conserved during evolution (1–3). They function essentially by controlling the timing of activation and the substrate specificity of a series of Cdks[4] that are sequentially activated during the cell cycle. Several cyclins and Cdks have been identified in mammalian cells. Specific cyclins bind to specific Cdks, thus activating their kinase activity. Each of these cyclin/Cdk complexes is activated at a specific point during the cell cycle and has a specific set of substrates (1–6).

G1 cyclins regulate the progression of cells through the G_1 phase of the cell cycle and drive entry into S phase. Three D-type cyclins, D1, D2, and D3, act at mid-G_1 by complexing with either Cdk4 or Cdk6 (1, 3, 7). Cyclin E acts in late G_1 by complexing with Cdk2 (8, 9).

Cyclin E is a nuclear protein originally isolated by screening human cDNA libraries for genes that could complement the loss of G1 cyclins in *Saccharomyces cerevisiae* (10, 11). The cyclin E/Cdk2 complex shows strong kinase activity shortly before cells enter S phase and leads to further phosphorylation of the pRb protein (12–14). The accumulation and binding of cyclin E to Cdk2 are not the only mechanisms of regulating cyclin E/Cdk2 kinase activity. Both positive and negative phosphorylation events, as well as association with specific inhibitory proteins, also contribute to this regulation. The activity of the cyclin E/Cdk2 complex is mainly regulated by members of a family of CDIs, which include p21^{Waf1} (also designated Cip1, Pic1, Sd1, and Cap20) and p27^{Kip1} (also called Ick and Pic2; reviewed in Refs. 2 and 15–17).

The accumulation of cyclin E and activation of the cyclin E/Cdk2 complex is a rate-limiting event for the G_1-to-S transition. In fact, antibodies to cyclin E inhibit entry into S phase when injected into cells during the G_1 phase of the cell cycle (18). On the other hand, overexpression of cyclin E accelerates the G_1-to-S transition, decreases cell size, and reduces the serum requirement for growth in human and rat fibroblasts (18–20). Although both cyclins D1 and E play a role in phosphorylating the Rb tumor suppressor protein in mid-late G_1, the functions of these two cyclins are not redundant. In fact, overexpression of cyclin D1, but not cyclin E, is associated with increased phosphorylation of the Rb protein (21).

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[2] Present address: Centro di Ricerche Oncologiche "Giovanni XXIII," Catholic University, Largo Francesco Vito 1, 00168 Rome, Italy.

[3] To whom requests for reprints should be addressed, at Columbia-Presbyterian Comprehensive Cancer Center, 701 West 168th Street, Room 1509, New York, New York 10032. Phone: (212) 305-6921; Fax: (212) 305-6889.

[4] The abbreviations used are: Cdk, cyclin-dependent kinase; CDI, cyclin-dependent kinase inhibitory protein; TGF, transforming growth factor; Rb, retinoblastoma.

Moreover, in contrast to the results obtained with antibodies to cyclin D1 (22), injection of anti-cyclin E antibodies arrests cells in G_1, even in Rb minus cells (18). In addition, the cyclin D1/Cdk4 complex can phosphorylate *in vitro* the pRb-related protein p107 but not the pRb-related p130 protein Rb2, whereas the contrary is true for the cyclin E/Cdk2 complex (23, 24).

During the past decade, advances made in our understanding of the cell cycle machinery have clearly indicated that disruption of the normal cell cycle is one of the most important alterations involved in cancer development (2, 3, 16). Because the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G_1 phase of the cell cycle, deregulated expression of the G1 cyclins and Cdks might cause loss of cell cycle control and thus enhance tumorigenesis.

Increased expression of multiple cyclin E-related proteins has been reported in several human malignancies (25, 26). In breast cancer cell lines, the increased expression of cyclin E has been associated with increased cyclin E-associated kinase activity (27). However, we reported recently that overexpression of a human cyclin E cDNA in the nontransformed mouse mammary epithelial cell line HC11 was associated with increased expression of the cell cycle-inhibitory protein $p27^{Kip1}$ and inhibition rather than stimulation of cell growth (28). These findings are in contrast with the above-mentioned results (18–20) with rat and human fibroblasts.

To further address the role of cyclin E in cell growth control and tumorigenesis, in the present study we analyzed, in parallel, the effects of cyclin E overexpression in two fibroblast cell lines and in three nontumorigenic mammary epithelial cell lines. We found that overexpression of the same cyclin E cDNA exerted cell-specific effects on various parameters, including doubling time, saturation density, plating efficiency, cell cycle distribution, cyclin E-associated kinase activity, and the level of expression of $p27^{Kip1}$. We also show that overexpression of cyclin E is not able to confer anchorage-independent growth in any of the cell lines analyzed, but it does modulate the responses of some of these cell lines to TGF-β. The implications of these findings are discussed.

Results

Generation of Derivatives That Stably Overexpress Cyclin E. To further address the role of cyclin E in cell cycle control and transformation, we analyzed the phenotypes of derivatives of four different cell lines that stably overexpress cyclin E and also compared the results obtained with derivatives of the HC11 mouse mammary epithelial cell lines, the properties of which we have described previously (28). The four new cell lines were Rat1 fibroblasts, NIH3T3 mouse fibroblasts, and 184B5 and MCF-10F nontransformed human mammary epithelial cells. These cell lines were transduced with the same human cyclin E cDNA expressed from a retroviral promoter used in our previous studies with the HC11 cell line (28). After selection for resistance to hygromycin (*hph*), pools of thousands of resistant colonies were obtained, both from the cultures infected with the PMV12-cycE construct and the cultures infected with the PMV12pl vector (vector control cells), and used for the studies described below.

Expression of the exogenous cyclin E gene was verified by Western blot analysis using either a polyclonal anti-cyclin E antibody or an antihuman cyclin E monoclonal antibody that specifically recognizes human cyclin E. All of the above-mentioned five cell lines used in the present studies expressed variable levels of the major endogenous cyclin E band, which was about M_r 55,000 in the rat and mouse cells and about M_r 52,000 in the human cells (Figs. 1 and 2; Refs. 18, 19, and 28). As reported previously (28), the polyclonal antibody mainly detected a M_r 50,000 band corresponding to the exogenous cyclin E in the overexpressor cells (Fig. 6 and data not shown), whereas the monoclonal antibody also detected a prominent exogenous M_r 42,000 band (Ref. 28; Figs. 1 and 2). As shown in Figs. 1 and 2, we used the monoclonal antibody to rule out the possibility that the different effects seen in the different cell lines might have been because of different levels of expression of the exogenous M_r 42,000 protein in the various cell lines. The polyclonal anti-cyclin E antibody, which only recognizes the M_r 50,000 exogenous cyclin E band, was used (see Fig. 6) because it enabled us to detect both the exogenous and the endogenous cyclin E proteins. We have previously reported the overexpression of cyclin E in HC11 and Rat1 cells (28).

Overexpression of the exogenous cyclin E cDNA was verified by Northern blot analysis, and nuclear localization of the cyclin E protein and its increased level in the derivatives of the five cell lines were confirmed by immunostaining with an anti-cyclin E antibody (data not shown).

It is of interest that in multiple transduction experiments with the 184B5 cells the number of hph^+ clones obtained after transduction with the PMV12-cycE construct was lower than the number obtained after transduction with the control PMV12pl construct. Furthermore, with the 184A1 nontumorigenic human mammary epithelial cell line (29), we were unable to obtain any hph^+ colonies that expressed the exogenous cyclin E (data not shown). These findings suggest that overexpression of this cyclin E cDNA was somewhat toxic to the 184B5 cell line and highly toxic to the 184A1 cell line. Moreover, the cyclin E overexpressing pools of the 184B5 cells showed only a moderate level of expression of the exogenous cyclin E bands, because the level was considerably lower than that obtained with the MCF-10F and Rat1 cell lines (Fig. 2). We also tried to obtain single clones of 184B5 cells that overexpressed the exogenous cyclin E. However, when we analyzed several hph^+-resistant clones for expression of cyclin E, we found that they expressed only the M_r 42,000 exogenous cyclin E (Fig. 2, last two lanes), which might correspond to a spliced form of cyclin E reported previously to be unable to activate Cdk2 (30). These findings are consistent with studies described below indicating that ectopic expression of this cyclin E cDNA in 184B5 cells inhibits their growth, thus resembling the situation with HC11 cells (28).

Effects of Cyclin E Overexpression on Cell Cycle Kinetics and Cell Growth. To evaluate the phenotypic effects of cyclin E overexpression, several parameters were examined, in parallel, in the vector control pools of the three cell

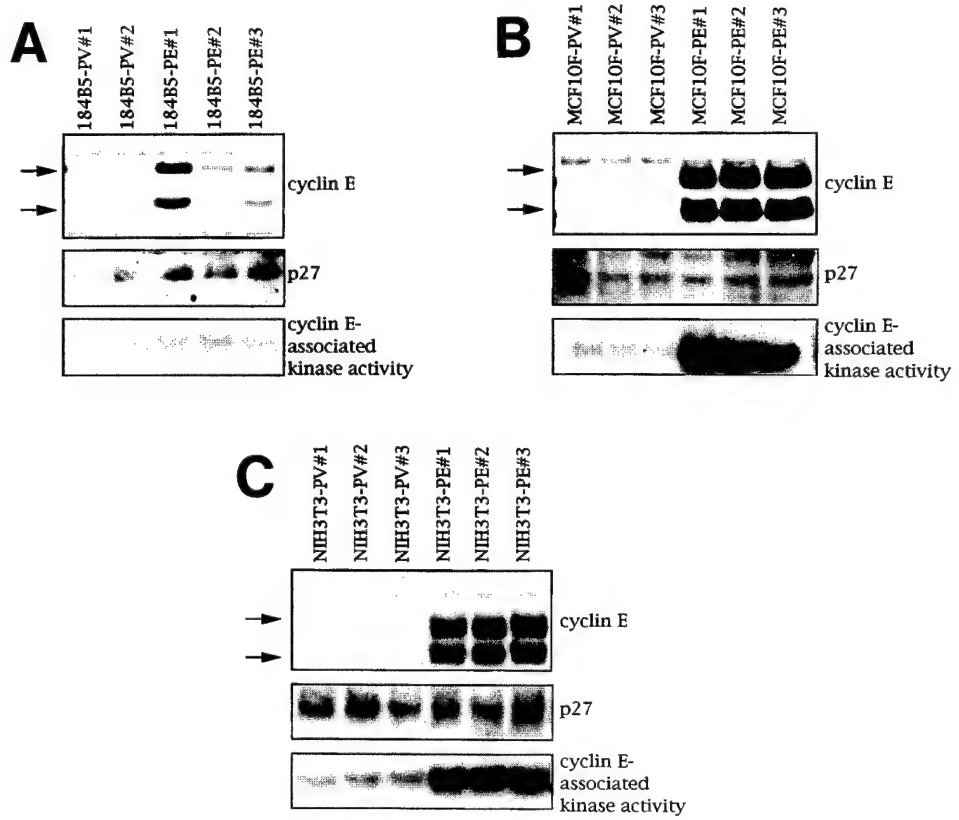

Fig. 1. Effects of stable overexpression of cyclin E in the 184B5 (*A*) and MCF-10F (*B*) human nontumorigenic mammary epithelial cell lines and in NIH3T3 (*C*) mouse fibroblasts. Exponentially growing cultures of vector control (*PV*) and cyclin E-overexpressing pools (*PE*) of each cell line were analyzed. For Western blot analyses, 50 μg of proteins in whole-cell lysates were resolved by 10% SDS-PAGE and transferred to an Immobilon membrane. Duplicate blots were probed with the anti-cyclin E (*top panels*) and the anti-$p27^{Kip1}$ (*middle panels*) antibodies, and immunoreactive bands were detected by enhanced chemiluminescence. The monoclonal antihuman cyclin E antibody was used for detecting cyclin E, because it recognizes both the M_r 50,000 and the M_r 42,000 bands corresponding to the exogenous cyclin E. *Arrows,* positions of the exogenous cyclin E bands. For the histone H1 kinase activity (*bottom panels*), 50 μg of whole-cell lysates were used for derivatives of the MCF-10F and NIH3T3 cells, and 100 μg were used for derivatives of the 184B5 cells. For additional details, see "Materials and Methods."

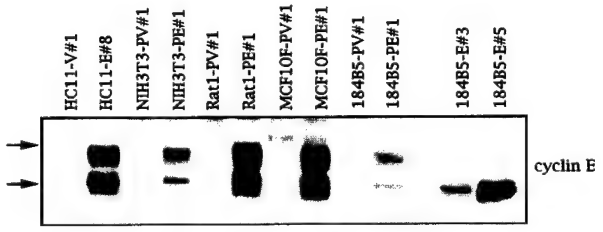

Fig. 2. Western blot analysis for human cyclin E proteins in various cell lines are shown. *Lanes 1* and *2* are previously described (28) vector control and cyclin E-overexpressor derivatives of the HC11 mouse mammary cell line, respectively. Also shown are vector control (*PV*) and representative cyclin E-overexpressing pools (*PE*) of NIH3T3, Rat1, MCF-10F, and 184B5 cells. The monoclonal antihuman cyclin E antibody was used for detecting cyclin E. *Arrows,* positions of the M_r 50,000 and M_r 42,000 bands corresponding to the exogenous cyclin E. The *last two lanes* are two single cyclin E-overexpressing clones (*E#3* and *E#5*) of the 184B5 cell line that express only the M_r 42,000 exogenous cyclin E protein.

lines, NIH3T3-PV, 184B5-PV, and MCF10F-PV, and in the corresponding cyclin E overexpressor pools, NIH3T3-PE, 184B5-PE, and MCF10F-PE (Table 1). The effects of cyclin E overexpression on cell cycle kinetics and cell growth have been reported previously in Rat1 cells (19, 28) and HC11 cells (28).

Overexpression of cyclin E in NIH3T3 cells had no effect on the distribution of cells in the cell cycle. Growth curves of monolayer cultures also indicated no appreciable differences in the doubling times or saturation densities between cyclin E-overexpressor derivatives of NIH3T3 cells and the corresponding vector control cells. However, the NIH3T3-PE cells showed a higher plating efficiency compared with the NIH3T3-PV cells (25% *versus* 14%; Table 1). These effects were associated with an increase in cyclin E-associated kinase activity, and no changes were observed in the cellular level of the $p27^{Kip1}$ protein (Fig. 1C).

The cyclin E-overexpressing derivatives of MCF-10F cells displayed a reduction in the percentage of cells in the G_1 phase (about 52% *versus* 62%) and an increase in the percentage of cells in S phase (about 15% *versus* 8%), when compared with the vector control cells (Table 1). In addition, the MCF10F-PE cells displayed a slightly shorter doubling time than the vector control cells and an increased saturation density (Table 1). Unexpectedly, the plating efficiency of MCF10F-PE cells was reduced when compared to MCF10F-PV cells (4.0% *versus* 6.6%). The cyclin E-associated kinase activity was markedly increased in the MCF10F-PE cells, and no change was observed in the level of the $p27^{Kip1}$ protein in these cells (Fig. 1*B*).

The cyclin E-overexpressing pools of the 184B5 cell line showed an increase in the percentage of cells in the G_1 phase of the cell cycle (about 65% *versus* 55%) and a reduction of cells in S phase (about 23% *versus* 30%) when compared with the corresponding vector control cells. These derivatives of 184B5 cells also displayed a longer doubling time, a lower saturation density, and decreased plating efficiency when compared with the vector control cells (Table 1).

Table 1 Effects of cyclin E overexpression on cell cycle distribution and growth properties in different cell lines

All assays were performed in triplicate, and all experiments were repeated at least twice. The data reported are the results of a typical experiment for a representative cell line. Individual points are the means of triplicate determinations. SD for individual points were less than 25% of the mean. Similar results were obtained in replicate experiments.

Cell line	D.T.[a] (h)	S.D.[b] ($\times 10^6$)	P.E.[c] (%)	G_0-G_1	S	G_2-M	A.I.G.[d] (%)	$p27^{Kip1e}$	Cyclin E kinase activity[e]
					(%)[f]				
Rat1-PV#1	17.0	4.0	14.0	58.8	30.8	10.3	0		
Rat1-PE#1	17.0	5.2	29.0	35.9	46.9	17.2	0	No change	Increase
MCF10F-PV#1	30.0	3.5	6.6	61.9	8.5	29.7	0		
MCF10F-PE#1	27.8	4.3	4.0	52.3	15.1	32.7	0	No change	Increase
NIH3T3-PV#1	15.0	2.7	14.0	55.1	34.3	10.6	0		
NIH3T3-PE#1	15.0	2.6	25.0	54.9	34.9	10.2	0	No change	Increase
184B5-PV#1	25.8	2.6	11.6	55.1	29.7	15.2	0		
184B5-PE#1	29.8	1.5	6.4	68.2	22.6	9.1	0		
184B5-PE#2	28.0	1.7	8.0	62.7	26.7	10.6	0	Increase	No change
HC11-V#1[g]	14.6	11.4	8.0	28.9	53.7	17.4	1.7		
HC11-E#8	18.0	6.6	4.2	43.5	45.9	10.6	0.1	Increase	No change

[a] D.T., doubling time, corresponds to the initial exponential phase of the cell growth.
[b] S.D., saturation density, represents the total number of cells per 35-mm well when the cultures reached a plateau.
[c] P.E., plating efficiency.
[d] A.I.G., anchorage-independent growth, expressed as colony-forming efficiency in soft agar.
[e] These results refer to the situation in the cyclin E-overexpressing derivatives of each cell line when compared to the corresponding vector control cells.
[f] Exponentially growing cultures of the indicated cell lines were analyzed by flow cytometry. The values represent the percentage of the total cell population in each phase of the cell cycle.
[g] Most of the data regarding the derivatives of HC11 cells have been reported previously (Cancer Res., 56 1389–1399, 1996). For additional details, see "Materials and Methods."

These differences, although not dramatic, were reproducible in multiple experiments and were observed in separately isolated pools of the cyclin E-overexpressing 184B5 cells (data not shown). Moreover, these changes were related to the level of expression of the exogenous cyclin E (compare the data on the 184B5-PE#1 and 184B5-PE#2 pools in Fig. 1*A* and Table 1). As reported previously for the mouse mammary epithelial cell line HC11 (28), when exponentially growing cells were analyzed for the amount of cyclin E-associated kinase activity, no significant increase was observed between the cyclin E-overexpressing 184B5 cells and the vector control cells. Exponentially dividing cultures of the cyclin E-overexpressing pools also displayed increased expression of the $p27^{Kip1}$ protein when compared with the control pools (Fig. 1*A*). As mentioned above, single clones of 184B5 cells isolated from cultures transduced with the cyclin E vector expressed only the M_r 42,000 cyclin E protein band (Fig. 2, last two lanes) and did not display an increase in cyclin E-associated kinase activity (data not shown).

Overexpression of Cyclin E Is Not Sufficient to Induce Anchorage-independent Growth. A recent report suggested that lack of activation of cyclin E-associated kinase activity is responsible for the G_1 block observed when untransformed human diploid fibroblasts are maintained in suspension rather than allowed to become adherent to a growth surface (31). This conclusion was based on the finding that activation of cyclin E/Cdk2, which is required for the G_1-to-S transition, was seen in late G_1 in adherent fibroblasts but not in fibroblasts maintained in suspension. On the other hand, in anchorage-independent transformed fibroblasts, the cyclin E/Cdk2 complex was activated in late G_1, even when the cells were grown in suspension. These investigators also reported that the lack of cyclin E/Cdk2 activity in the suspended untransformed fibroblasts was associated with increased expression of $p27^{Kip1}$ (31).

It was of interest, therefore, to examine the responses of our derivatives of Rat1 and NIH3T3 fibroblasts that stably overexpress cyclin E to growth in suspension, using a protocol reported previously (32). Asynchronous adherent cultures of Rat1-PV, Rat1-PE, NIH3T3-PV, and NIH3T3-PE cells were trypsinized and replated in parallel onto nontreated tissue culture plastic dishes (adherent cultures) and onto agar-coated dishes (nonadherent cultures), as described in "Material and Methods." After 48 h, cells from both types of cultures were collected, and their cell cycle distribution was analyzed by flow cytometry. Representative results are shown in Fig. 3. As expected, with the adherent cultures of all four cell lines, which were growing exponentially, cells were distributed throughout the cell cycle (Fig. 3*B*). About 55% of the NIH3T3-PV and NIH3T3-PE cells were in G_1. The respective values for Rat1-PV and Rat1-PE were 59 and 36%, respectively.

The suspension cultures gave very different results (Fig. 3*A*). About 85% of the NIH3T3-PV, NIH3T3-PE, and Rat1-PV cells arrested with a 2N DNA content after having been grown in suspension for 48 h, indicating cell cycle arrest at some point in G_0 or G_1. On the other hand, when the Rat1-PE cells were grown in suspension for 48 h the distribution of cells in the G_0-G_1, S and G_2-M phases of the cells was 53.4, 37.1, and 9.5%, respectively. Therefore, after growth in suspension for 48 h, the Rat1 cyclin E-overexpressing cells had not undergone significant growth arrest. However, the Rat1-PE cells did show a progressive accumulation of cells in G_0-G_1 at later time points; thus, after 96 h in suspension, about 80% of the cells were in G_0-G_1 (data not shown).

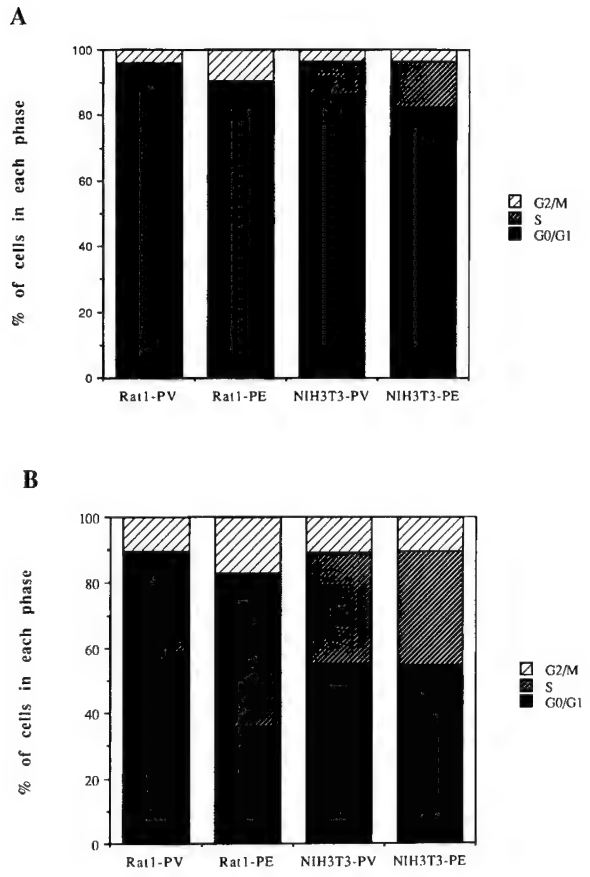

Fig. 3. Accumulation of cells in G_0-G_1 in the absence of cell adherence. In *A*, asynchronous adherent cultures of the indicated vector control (*PV*) and cyclin E-overexpressing pools (*PE*) were trypsinized and replated onto agar-coated dishes. After 48 h, the suspended cells were collected, and their cell cycle distribution was analyzed by flow cytometry. In *B*, the cell cycle distribution of parallel adherent cultures of the same cell lines is shown for comparison. Cells remained >90% viable after 2 days in suspension.

To extend this analysis, we analyzed synchronized cultures. Adherent monolayer cultures of Rat1-PV and Rat1-PE cells were arrested in G_0 for 72 h by serum starvation, trypsinized, and then replated into untreated and agar-coated tissue culture dishes. After serum re-stimulation, the cell cycle distribution of both cultures was analyzed by flow cytometry at various times thereafter. As shown in Fig. 4, adherent cultures of both Rat1-PV and Rat1-PE were in S phase after about 14 h, and thereafter, proceeded through G_2-M and reentered G_1. On the other hand, the progression from G_0 to S was markedly reduced in nonadherent cultures of both Rat1-PV and Rat1-PE cells (Fig. 4). It is of interest that whereas most of the Rat1-PV cells were cell cycle arrested after 24 h of growth in suspension, *i.e.*, >85% of these cells were in G_1, with Rat1-PE cells, 69% of the cells were in G_1 and 22% were in S phase after 24 h. However, when kept in suspension for 72 h, >85% of the Rat1-PE cells accumulated in G_0-G_1 (data not shown).

We also examined the ability of the above described cell lines to undergo cell division in suspension culture by doing serial cell counts for up to 48 h after the cells were transferred to agar-coated plates (Fig. 5). It was apparent that the NIH3T3-PV, NIH3T3-PE, and Rat1-PV cells did not show a significant increase in cell number. However, the Rat1-PE cultures underwent about one doubling of the cell population within the first 24 h but did not increase in number thereafter. These data, together with the cell cycle data in Fig. 4, indicate that overexpression of cyclin E in NIH3T3 cells does not confer the ability to grow in suspension. Overexpression of cyclin E in Rat1 cells does allow them to undergo about one cell division but does not allow continuous cell replication in suspension cultures.

To extend these results, we tested whether the cyclin E-overexpressing derivatives of the various cell lines used in this study had an enhanced ability to form colonies in soft agar when compared with the vector control cells. However, neither the vector control or the cyclin E derivatives of the Rat1, NIH3T3, MCF-10F, or 184B5 cells were able to form colonies in soft agar, even in medium with a high concentration of serum (up to 30%; Table 1 and data not shown). We reported previously that HC11 cells are able to grow in soft agar, although with a very low efficiency, and this ability was actually reduced in the cyclin E-overexpressor derivatives (28). Therefore, overexpression of cyclin E does not confer anchorage-independent growth to any of the five cell lines listed in Table 1.

It was also of interest to examine cyclin E-associated kinase activity in extracts obtained from adherent and nonadherent cultures of Rat1-PV and Rat1-PE cells. Adherent exponentially growing cultures of Rat1-PE cells showed about a 5-fold higher cyclin E-associated kinase activity than the comparable vector control cells. However, after growth in suspension for 48 h, both cell lines showed a marked decrease in cyclin E-associated kinase activity, although this activity remained slightly higher in the cyclin E-overexpressing cells (Fig. 6*A*). The decrease in cyclin E-associated kinase activity was not associated with a decrease in the level of the cyclin E protein, but as reported previously for KD and IMR90 cells (31), growth in suspension led to a marked increase in the level of expression of the $p27^{Kip1}$ protein (data not shown).

We also analyzed cyclin E expression and cyclin E-associated kinase activity in adherent and nonadherent cultures of G_0-synchronized Rat1-PV and Rat1-PE cells. Serum-starved cells were restimulated with serum (as described in Fig. 4), and protein extracts were prepared from cells collected at various times after the addition of serum. In adherent cultures, the level of expression of the endogenous cyclin E protein in both Rat1-PV and Rat1-PE cells peaked at about 14 h, corresponding to the G_1-S boundary, and was decreased at 20 h (Fig. 6*B*). In nonadherent cultures of both cell lines, the level of expression of the endogenous cyclin E was increased at 14 h but remained high at 20 h. The latter finding suggested that the nonadherent cells arrested in late G_1, after the induction of cyclin E. As expected, throughout these studies, there was constitutive expression of the exogenous M_r 50,000 cyclin E protein in the Rat1-PE cells (Fig. 6*B*). In the above studies, we were surprised to observe an appreciable amount of the endogenous cyclin E protein in the cells

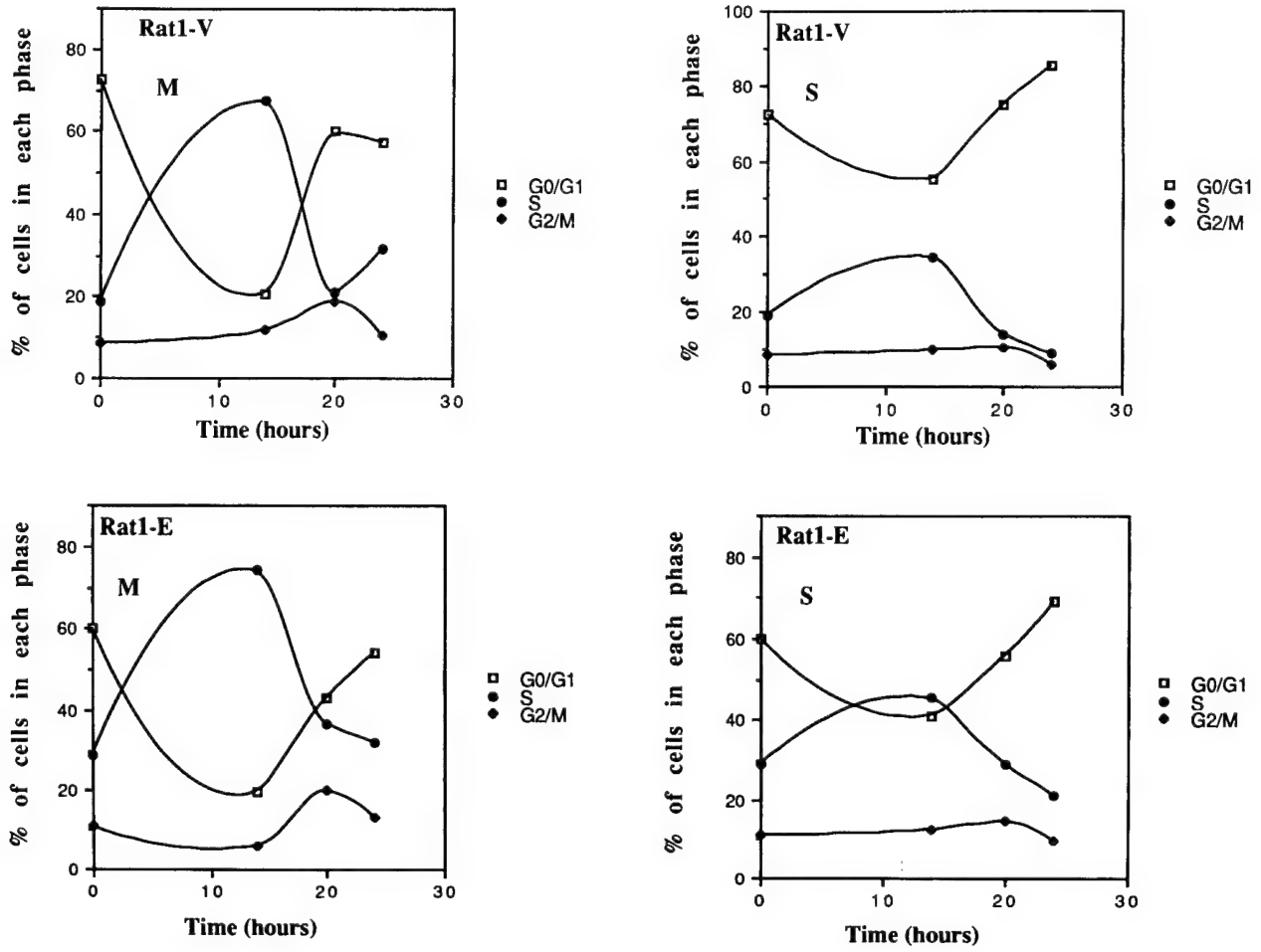

Fig. 4. Flow cytometric analysis of cell cycle progression in nonadherent cultures of vector control (Rat1-V) and cyclin E-overexpressing (Rat1-E) pools of Rat1 cells. Adherent cultures of cells were first synchronized in G_0 by serum starvation and then trypsinized and transferred in complete medium-containing serum into agar-coated dishes (*S*). Cells were subsequently collected at the indicated times, stained with propidium iodide, and analyzed by flow cytometry as described in "Materials and Methods." Parallel adherent cultures (*M*) of the same cell lines are shown for comparison. Cell viability in all cultures was >90% (by trypan blue exclusion) throughout the experiment. Similar results were obtained with several independent pools. *M*, monolayer; *S*, suspension.

at time 0 (Fig. 6*B, top panel, Lanes 1* and *6*). This finding was reproducible in several experiments. It might be because of the incomplete accumulation of the cells in G_0 (Fig. 4). However, the presence of cyclin E in fibroblasts arrested in G_0 by serum starvation has also been observed by other investigators (33, 34).

Assays for cyclin E-associated kinase activity indicated that this activity was maximal at about 14 h in the adherent cultures of both Rat1-PV and Rat1-PE cells, thus paralleling the level of expression of the cyclin E protein. Despite the high level of cyclin E protein, the cyclin E-associated kinase activity was much lower in the nonadherent cultures than the comparable adherent cultures. Therefore, in nonadherent cultures of Rat1-PV cells, there was only a slight increase in cyclin E-associated kinase activity at 14 h, and this activity could not be detected at 20 h. The induction of cyclin E-associated kinase activity was also reduced in the nonadherent cultures of Rat1-PE cells, when compared with monolayer cultures of the same cells. However, this activity was always higher in the nonadherent cultures of Rat1-PE cells than in nonadherent cultures of Rat1-PV cells, even at 20 h (Fig. 6*B*).

We then examined the level of the CDI $p27^{Kip1}$ under the same conditions. In the adherent cultures of both the Rat1-PV and Rat1-PE cells, the level of this protein at time 0 was relatively high (reflecting the effect of serum starvation). In both cell types, it was markedly decreased at 14 and 20 h (Fig. 6*B*). In the nonadherent cultures of both cell types, the level of $p27^{Kip1}$ remained high at both 14 and 20 h (Fig. 6*B*). This finding is consistent with previous studies on nonadherent KD and IMR90 cells (31).

These data demonstrate that although cyclin E overexpression is associated with an increased cyclin E-associated kinase activity in asynchronous adherent cultures of Rat1 cells, this increase is not sufficient to override the inhibition of cyclin E/Cdk2 activity that occurs in nonadherent cells. This apparently reflects the high levels of induction of $p27^{Kip1}$ in the nonadherent cultures.

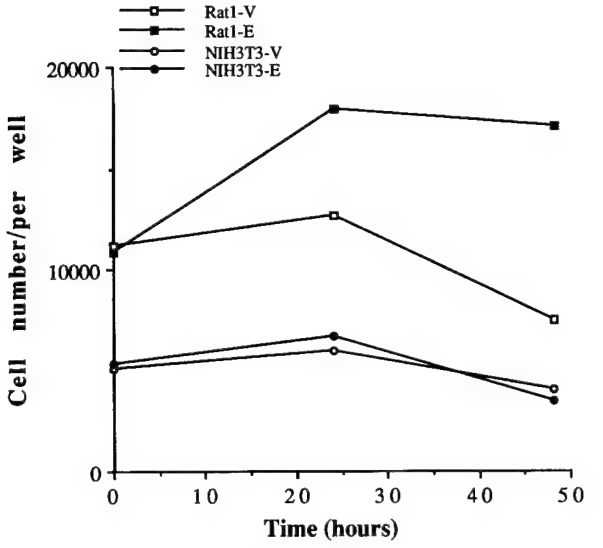

Fig. 5. Growth curves of nonadherent cultures of vector control (Rat1-V and NIH3T3-V) and cyclin E-overexpressing (Rat1-E and NIH3T3-E) pools of Rat1 and NIH3T3 cells. Asynchronous adherent cultures of each cell line were trypsinized, and the cells were replated into agar-coated dishes in complete growth medium containing serum. Cell numbers were determined in triplicate at the indicated times using a Coulter counter. No further growth was observed at later time points (data not shown). The data points are the means of triplicate assays that varied by less than 10% and represent the numbers of cells per 35-mm dishes. Cell viability in all cultures was >90%. Similar results were obtained with several independent pools.

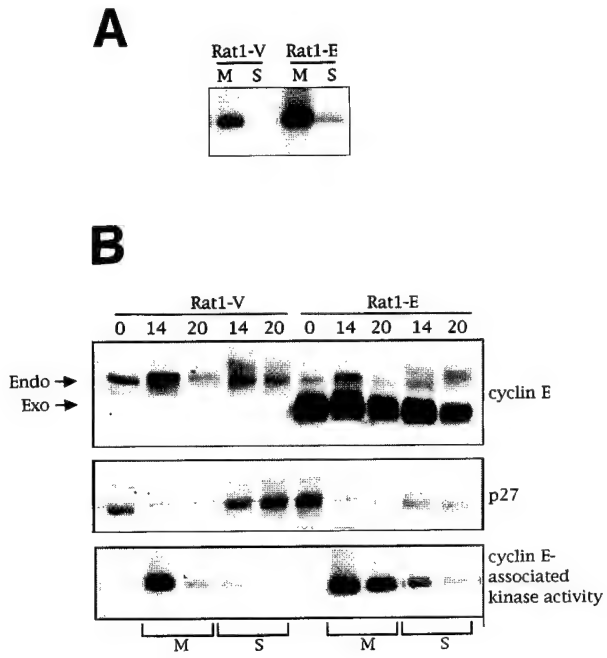

Fig. 6. Effects of cell adhesion on the level of expression of cyclin E and $p27^{Kip1}$ and on cyclin E-associated kinase activity in vector control (Rat1-V) and cyclin E-overexpressing (Rat1-E) pools of Rat1 cells. In *A*, 50 μg of whole-cell lysates from asynchronous adherent (*M*) and suspended (*S*) cultures of the indicated cell lines were used to analyze the histone H1 kinase activity of anti-cyclin E immunoprecipitates. In *B*, adherent cultures were first synchronized in G_0-G_1 by serum starvation. They were then trypsinized and replated as adherent (*M*) or suspension (*S*) cultures in the presence of serum. At the indicated times, cells were collected, and extracts were examined by Western blot analysis for cyclin E (*top panel*) and $p27^{Kip1}$ (*middle panel*) and for cyclin E-associated kinase activity using histone H1 as the substrate (*bottom panel*) as described in "Materials and Methods." The polyclonal anti-cyclin E antibody, which only recognizes the M_r 50,000 exogenous cyclin E band, was used for this experiment because it enabled us to detect both the exogenous and the endogenous cyclin E proteins. *Left*, positions of the endogenous (*Endo*) and the exogenous (*Exo*) cyclin E proteins. Similar results were obtained with several independent pools.

Overexpression of Cyclin E Modulates the Response of Some of These Cell Lines to TGF-β. TGF-β is a multifunctional cytokine that can affect cell proliferation and differentiation in a variety of cell types. It is a potent growth inhibitor of many types of normal epithelial cells, both in cell culture and *in vivo* (35). In some cell lines, the growth-inhibitory effects of TGF-β are associated with inhibition of cyclin E/Cdk2 kinase activity, although the mechanisms that mediate this inhibition appear to be different in different cell types (reviewed in Refs. 36 and 37). Therefore, it was of interest to determine whether overexpression of cyclin E modified the effects of TGF-β on cell proliferation.

HC11 cells are known to be responsive to the growth-inhibitory effects of TGF-β1 in terms of DNA synthesis and cell growth (38). Control HC11 cells and cyclin E-overexpressing derivatives were incubated in medium with or without TGF-β1 (5 ng/ml) for 48 h, and the cultures were then pulse labeled with [^{3}H]thymidine to measure DNA synthesis (see "Materials and Methods"). Fig. 7*A* indicates that TGF-β1 inhibited [^{3}H]thymidine incorporation by about 50% in both the parental and vector control cells. However, DNA synthesis was not inhibited in the HC11-E#8 cells that express a high level of cyclin E (Fig. 2; Ref. 28) but was inhibited by about 34% in the HC11-E#12 cells that express only a moderate level of cyclin E (28). Similar results were obtained after incubation with TGF-β1 for 24 or 72 h (data not shown). Growth curves in the presence of 5 ng/ml TGF-β1 with these four derivatives of HC11 cells confirmed that overexpression of cyclin E partially protected HC11 cells from inhibition by TGF-β1 (data not shown).

Overexpression of cyclin E in the 184B5 cell line partially protected them from inhibition of [^{3}H]thymidine incorporation by TGF-β (Fig. 7*B*) and also from inhibition of cell growth by TGF-β (data not shown), but this protection was not as great as that obtained with HC11 cells (Fig. 7*A*). This is probably because of the lower level of expression of the exogenous cyclin E in the 184B5 cells. Similar results were obtained after incubation with TGF-β1 for 24 or 72 h (data not shown). Under similar conditions, TGF-β1 caused about a 50% inhibition of [^{3}H]thymidine incorporation with both the parental and vector control of MCF-10F cells. However, with this cell line, a similar level of inhibition was observed with the cyclin E-overexpressing derivatives (Fig. 7*C*). Growth curves also indicated that TGF-β1 caused a similar extent of inhibition in both the vector control and cyclin E-overexpressing derivatives of MCF-10F cells (data not shown).

TGF-β1 stimulated [^{3}H]thymidine incorporation about 2.6-fold in both the Rat1 and NIH3T3 vector control fibroblasts. This is consistent with the known growth-stimulatory effects of TGF-β on fibroblasts (35, 39). This stimulation was re-

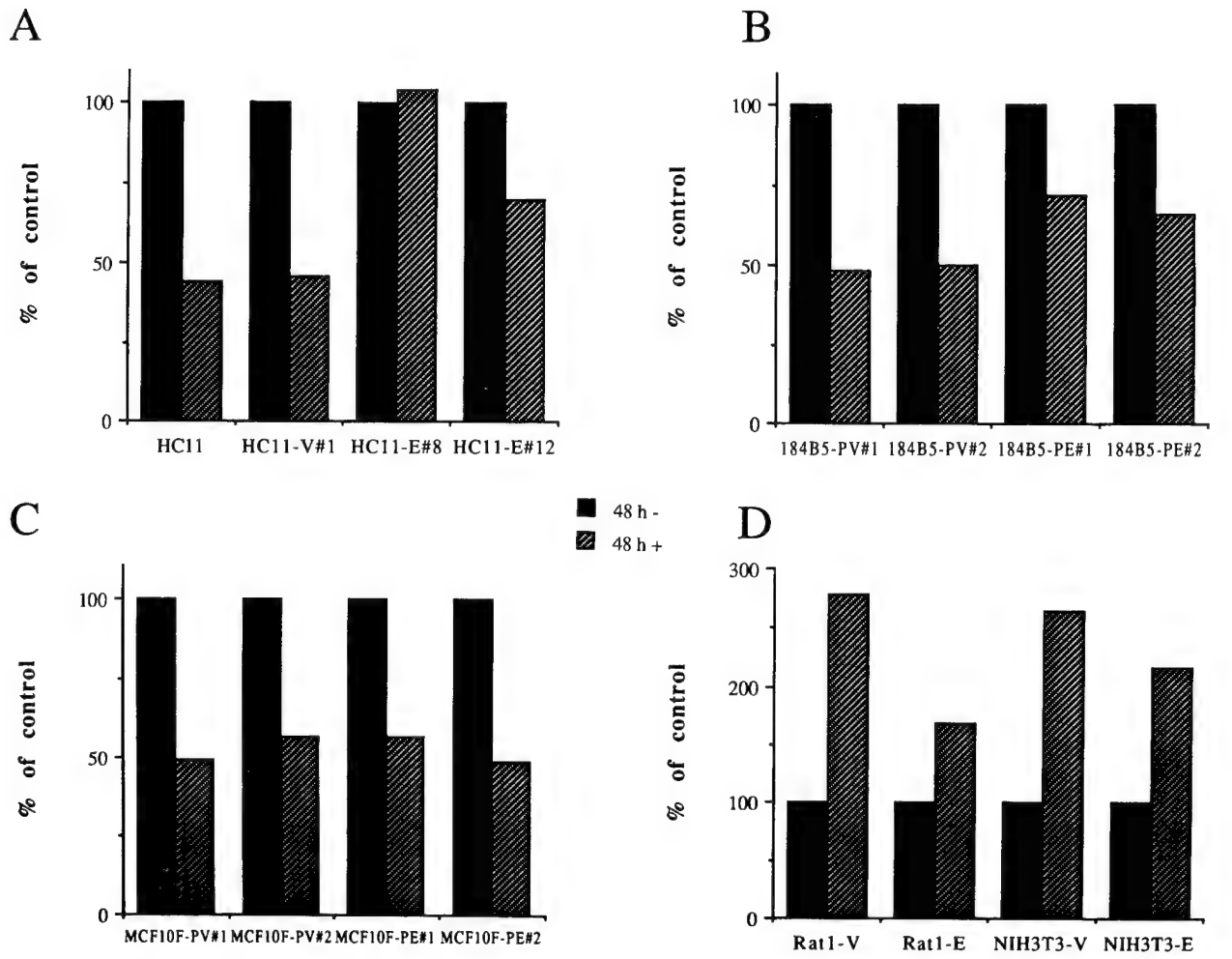

Fig. 7. Effects of stable overexpression of human cyclin E on the responses of different cell lines to TGF-β. Representative vector control (designated *V* or *PV*) and cyclin E-overexpressing derivatives (designated *E* or *PE*) of the indicated cell lines were plated in 24-well plates (10^4 cells/well), grown for 24–36 h, and then incubated in the absence or presence of TGF-β1 for 48 h (as described in "Materials and Methods") before adding [^{3}H]thymidine for 1 h. Values are expressed as the percentage of [^{3}H]thymidine incorporation into the acid-insoluble fraction in the presence of TGF-β1 (48 h+) *versus* the corresponding values obtained in the absence of TGF-β1 (48 h−), considered as 100%. Similar results were obtained after treating cells with TGF-β1 for 24 and 72 h (data not shown). Single clones were used for the HC11 cells, whereas pools of cells were used for all of the other cell lines. Individual points are the means of triplicate determinations. SDs for individual points were less than 20% of the mean. Similar results were obtained in three replicate experiments.

duced to 1.7- and 2.3-fold, respectively, in the corresponding cyclin E-overexpressing derivatives (Fig. 7*D*). Similar results were obtained after incubation with TGF-β1 for 24 or 72 h (data not shown). Therefore, overexpression of cyclin E can dampen the usual growth-inhibitory effects of TGF-β on epithelial cells and also dampen the usual growth-stimulatory effects of TGF-β on fibroblasts.

Discussion

Cyclin E overexpression has been observed in a variety of human cancers, including breast cancer, and it has been proposed that cyclin E might play an important role in tumor development, thus acting as an oncogene (25–27, 40). However, little is known about the actual role of increased expression of cyclin E in cell transformation and tumorigenesis. Therefore, it was of interest to examine, in parallel, the phenotypic effects of overexpressing an exogenous cyclin E in a series of cell lines.

The present study demonstrates that overexpression of the same cyclin E cDNA in different cell lines causes markedly different effects on cell cycle kinetics and growth parameters. These data are summarized in Table 1. Some of the data on HC11 cells were published previously (28) and are included for comparison. Overexpression of cyclin E was associated with a shortening of the G_1 phase of the cell cycle in Rat1 but not in NIH3T3 fibroblasts. Derivatives of both of these cell lines showed approximately similar increases in cyclin E-associated kinase activity, thus suggesting that high cyclin E/Cdk2 activity is necessary but not sufficient to drive entry into S phase. Both cell lines showed an increase in plating efficiency, but the saturation density was increased only in Rat1-PE cells when compared to the corresponding

vector control cells. Therefore, cyclin E overexpression caused an overall stimulation of growth in both of the fibroblast cell lines, but the characteristics and the magnitude of this stimulation were different. In mammary epithelial cells, increased expression of cyclin E was associated with a reduced percentage of cells in the G_0-G_1 phase of the cell cycle in the derivatives of the MCF-10F cells, whereas the cyclin E-overexpressing derivatives of 184B5 and HC11 cells displayed an increase in the percentage of cells in the G_0-G_1 phase of the cell cycle, and this was associated with a reduction of cells in the S phase. Cyclin E overexpression caused an overall stimulation of cell growth in MCF-10F cells but an inhibition of growth in 184B5 and HC11 cells. Unlike the situation in fibroblasts, these differences were directly related to the effects of cyclin E overexpression on cyclin E/Cdk2 activity. Therefore, cyclin E-associated kinase activity was increased in the MCF-10F but not in the HC11 and 184B5 derivatives. Derivatives of the latter two cell lines also displayed an increase in the level of expression of $p27^{Kip1}$, but the level of this protein was not increased in the cyclin E-overexpressing derivatives of MCF-10F, NIH3T3, and Rat-1 cells (Figs. 1 and 6). None of the cyclin E-overexpressing derivatives examined in this study displayed morphological evidence of malignant transformation or formed transformed foci in monolayer cultures.

It is of interest that the three cell lines, Rat1 fibroblasts, NIH3T3 fibroblasts, and MCF-10F cells, in which overexpression of cyclin E resulted in growth stimulation (by one or more parameters), also displayed increased cyclin E/Cdk2 kinase activity (Table 1). On the other hand, in the two cell lines 184B5 and HC11, in which cyclin E overexpression paradoxically caused an increase in the G_0-G_1 phase and an inhibition of cell growth, there was no increase in cyclin E/Cdk2 kinase activity, and there was increased cellular levels of $p27^{Kip1}$ protein. These differences among the cell types could not be explained simply by differences in the level of expression of the exogenous cyclin E cDNA (Fig. 2). Thus, although the 184B5 derivatives expressed a relatively low level of the exogenous cyclin E, their growth was inhibited, whereas the derivatives of MCF-10F and Rat1 cells expressed high levels of the exogenous cyclin E, and their growth was actually stimulated, although a comparable high level of exogenous cyclin E inhibited the HC11 cells (28). It would appear that in some, but not in all cell lines, increased expression of cyclin E can induce increased levels of the $p27^{Kip1}$ protein, which presumably accounts for the inhibitory effects seen in the latter cell lines. This effect is specific for $p27^{Kip1}$ because overexpression of cyclin E in the HC11 or MCF-10F cells and in Rat1 fibroblasts was not associated with altered levels of the CDIs $p21^{Waf1}$ and $p57^{Kip2}$ or cyclins D1 and A (28).[5] We have suggested previously that the increase in $p27^{Kip1}$ might reflect the existence of a homeostatic regulatory mechanism in which the set-point varies in different cell types (28). It may be of general relevance, because we have found that several human esophageal cancer cell lines,[5] breast cancer cell lines (28), and primary breast cancer cell lines,[5] express high levels of $p27^{Kip1}$ protein. The actual significance of this finding and the mechanism by which $p27^{Kip1}$ expression is induced is not known. It is of interest, however, that although the level of expression of $p27^{Kip1}$ in the three cyclin E-overexpressing pools of 184B5 cells was reproducibly higher than in control cells, it did not correlate quantitatively with the corresponding level of the exogenous cyclin E. In our previous studies on HC11 cells (28), we also found that although overexpression of cyclin E was associated with increased expression of $p27^{Kip1}$, there was not a proportional relationship between these two events. The significance of this finding is still unclear. It may be the effects of cyclin E overexpression on $p27^{Kip1}$ expression are not direct and because other still unknown factors influence the effects of cyclin E overexpression on $p27^{Kip1}$ expression. In studies on cyclin E-overexpressing derivatives of HC11 cells, we have found that the increased level of $p27^{Kip1}$ protein is not associated with an increase in $p27^{Kip1}$ mRNA, suggesting regulation at the protein level. Because recent studies indicate that the levels of $p27^{Kip1}$ protein can be regulated via a ubiquitin-dependent degradation pathway (41), it is possible that increased levels of cyclin E might interfere with this degradative mechanism in certain cell types. Indeed, in preliminary studies, we have found that the half-life of the $p27^{Kip1}$ protein is increased in the cyclin E-overexpressing HC11 cells. Studies are in progress to further examine the precise molecular mechanisms.

Recently, Fang *et al.* (31) reported the adhesion-dependence of cyclin E/Cdk2 activity in the KD and IMR90 untransformed human diploid fibroblast cell lines. They found that cyclin E/Cdk2 was activated in late G_1 in attached fibroblasts but not in fibroblasts maintained in suspension. In contrast, in the HUT12 cell line, an anchorage-independent, chemically transformed variant of KD cells, the cyclin E/Cdk2 complex was activated in late G_1 regardless of adhesion conditions. The lack of cyclin E/Cdk2 activity in suspended cells was associated with an increased expression of the Cdk2 inhibitors $p27^{Kip1}$ and $p21^{Waf1}$. Interestingly, these authors reported that the levels of expression of these Cdk2 inhibitors were comparable in both normal and transformed fibroblasts. Moreover, the levels of these two proteins increased in suspended cultures of transformed fibroblasts, as in the case of the normal fibroblasts. The maintenance of cyclin E/Cdk2 activity in suspended cultures of transformed fibroblasts was attributed to the higher levels of cyclin E, Cdk2, and the cyclin E/Cdk2 complex in these cells when compared to their normal counterparts. The authors concluded that the increased expression of cyclin E and Cdk2 may be important in the oncogenic transformation of HUT12 cells and suggested that the increased level of cyclin E might contribute to the anchorage-independent proliferation of tumor cells. Cyclin E overexpression has been reported in several human tumors, and its level has been related to tumor stage and grade in breast cancer (26). Moreover, it has been shown that the deregulated expression of cyclin E in some breast cancer cell lines is associated with an increased cyclin E-associated kinase activity, which unlike normal cells, is present constitutively and remains high throughout the cell cycle (27). Therefore, it was possible that cyclin E overex-

[5] Unpublished data.

pression contributed to tumor development by inducing anchorage-independent growth, which is a hallmark of transformed cells.

However, the present studies indicate that cyclin E overexpression is not sufficient *per se* to overcome the G_1 block observed in cells forced to grow in suspension. Thus, the growth of the cyclin E overexpressor NIH3T3-PE cells, like the vector control cells, was readily arrested in G_1 when they were deprived of adhesion. This G_1 block was delayed in the cyclin E-overexpressing derivatives of Rat1 cells, compared with vector control cells, because Rat1-PE cells showed a gradual and more delayed accumulation of cells in G_1 when kept in suspension culture. The limited G_1-S transit observed with the cyclin E-overexpressing derivatives of Rat1 cells was confirmed by doing growth curves on the cells in nonadherent cultures. Rat1-PE cells showed a significant increase in cell number after the first 24 h but no further increase at later time points, whereas no increase in cell number was observed in nonadherent cultures of Rat1-PV, NIH3T3-PV, and NIH3T3-PE cells (Fig. 5). These results suggest that overexpression of cyclin E in Rat1 fibroblasts, but not in NIH3T3 cells, confers only a very limited capacity for anchorage-independent growth, but it is not sufficient to completely override the adhesion requirement for continuous growth. Moreover, none of the cell lines used in this study were able to form colonies in soft agar after ectopic expression of cyclin E. Therefore, our data are not consistent with the suggestion of Fang *et al.* (31) that lack of cyclin E/Cdk2 kinase activity is the major factor responsible for the cell cycle block observed when nontransformed cells are grown in suspension. Our findings are, however, consistent with those of Guadagno *et al.* (42), indicating that lack of activation of cyclin A-associated kinase activity plays a critical role in this process. These investigators found that the expression of cyclin A in late G_1 was dependent on cell adhesion and that ectopic expression of cyclin A resulted in anchorage-independent growth in NRK fibroblasts. The fact that we found high levels of cyclin E in the arrested cells confirms that the block in cell cycle progression occurs at some point in late G_1 subsequent to cyclin E induction, probably before the induction of cyclin A and cyclin A/Cdk2 activity. It is, of course, possible that the lack of either cyclin E- or cyclin A-associated kinase activity is sufficient to cause the G_1 block observed in cells deprived of adhesion. Because our cyclin E overexpressor derivatives expressed levels of cyclin E that were often similar to those in human tumor cell lines (28) and the former cells were not anchorage independent, it seems likely that multiple events, in addition to cyclin E overexpression, contribute to the anchorage-independent growth observed with tumor cells. During the preparation of this manuscript, Zhu *et al.* (34) also reported that overexpression of cyclin E is not sufficient to induce anchorage-independent growth in human fibroblasts and also provided evidence that multiple events regulate anchorage-dependent growth.

It is well known that TGF-β is a complex regulator of cell growth (35). It displays activity on a great variety of cells and is able to either stimulate or inhibit their replication, depending on the cell type. The effects of TGF-β on the proliferation of several cell lines, both in terms of growth stimulation and growth inhibition, have been associated with the ability of this growth factor to interfere with the activity of the cyclin E/Cdk2 complex (reviewed in Refs. 36 and 37). Therefore, TGF-β causes a G_1 growth arrest in a variety of epithelial cell lines by inhibiting activation of the cyclin E/Cdk2 complex, although the mechanisms responsible for this inhibition appear to be different in different cell types and include: reduced expression of cyclin E; reduced expression of Cdk4; increased expression of p15; and increased expression of $p27^{Kip1}$ or $p21^{Waf1}$ (36, 37, 43). On the other hand, TGF-β stimulates the growth of fibroblast cell lines, and in some of these cell lines, this effect is associated with increased activity of the cyclin E/Cdk2 complex because of down-regulation of $p27^{Kip1}$ (44). It was of interest, therefore, to analyze whether overexpression of cyclin E was able to affect the responses of these cells to TGF-β in our model systems.

Indeed, in the present studies, we obtained preliminary evidence that overexpression of cyclin E interferes with the responsiveness of cells to TGF-β-mediated regulation of cell growth. Thus, in fibroblasts, in which TGF-β stimulates cell growth by activating cyclin E/Cdk2 activity (44), an increase in the basal level of cyclin E and cyclin E-associated kinase activity reduced the further stimulation of cell growth observed after exposure to TGF-β (Fig. 7*D*). The results obtained with the mammary epithelial cell lines were also of interest. Overexpression of cyclin E had no effect on TGF-β-mediated inhibition of growth in MCF-10F cells, but overexpression of cyclin E in HC11 and 184B5 cells reduced their responses to TGF-β-mediated inhibition of growth and DNA synthesis. This loss of inhibition was proportional to the level of expression of the exogenous cyclin E in both the HC11 and 184B5 cell lines and was not because of autonomous expression of TGF-β because immunoblot analysis with a pan-specific TGF-β antibody (R&D Systems, Minneapolis, MN) failed to detect TGF-β expression in either the control or the cyclin E overexpressor derivatives of these two cell lines (data not shown). Furthermore, the conditioned medium from the cyclin E-overexpressing cells had no effect on the rate of DNA synthesis or growth of the vector control cells, thus excluding that the cyclin E-overexpressing cells produce a soluble growth inhibitor (data not shown). Because there is evidence that TGF-β inhibits the growth of epithelial cell lines by reducing cyclin E/Cdk2 activity, the decreased inhibition of growth by TGF-β in the derivatives of HC11 and 184B5 cells may reflect the fact that they express increased levels of $p27^{Kip1}$ and already display decreased cyclin E/Cdk2 kinase activity. On the other hand, the decreased stimulation of growth by TGF-β in derivatives of fibroblasts that express increased levels of cyclin E may be because their high level of cyclin E kinase activity reduces the margin for a further TGF-β-mediated increase of this activity.

Regardless of the specific molecular mechanisms, the increased resistance to the TGF-β-mediated inhibition of cell growth seen with cyclin E-overexpressing derivatives of two mammary epithelial cell lines might be relevant to tumor development *in vivo*. This property could give a selective growth advantage to the cyclin E-overexpressing cells, although they grow more slowly than the parental cells. It is

tempting to speculate, therefore, that, at least in some cases, the frequent dysregulation of cyclin E in human tumor cells (26, 40) confers on these cells a selective advantage, not because it accelerates their cell cycle progression but because it increases their resistance to inhibition by TGF-β or other host factors. This is consistent with the observation that overexpression of cyclin E in primary tumors is not always associated with increased cell proliferation *in vivo* (26).

In conclusion, this report provides further evidence that the phenotypic effects of cyclin E overexpression are highly dependent on cell context and might depend on the interaction with other, not yet defined, pathways of cell regulation. Furthermore, we have also shown that overexpression of cyclin E in nontransformed fibroblasts or epithelial cells is not sufficient to confer anchorage-independent growth, although it might act in concert with other factors to cause this effect. Finally, we have demonstrated that overexpression of cyclin E can dampen the growth stimulatory effects of TGF-β in fibroblasts and the growth-inhibitory effects of TGF-β in mammary epithelial cells.

Although the underlying molecular mechanisms remain to be elucidated, the findings in this report indicate that it may not be possible to predict the specific phenotypic effects of increased expression of cyclin E in diverse human tumors, simply by measuring only the level of the cyclin E protein. Cell context also plays a major role presumably because of variations between cell types in diverse pathways that interact with the cyclin E/Cdk2 pathway.

Materials and Methods

Cells and Cell Culture. The origin and cell culture conditions of the normal human and mouse mammary epithelial cell lines 184B5 and HC11, respectively, and of the Rat1 fibroblasts have been described previously (28, 29). The MCF-10F, spontaneously immortalized human mammary epithelial cell line (45) was routinely cultured in a 1:1 (v/v) mixture of DMEM and Ham's F-12 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 20 ng/ml epidermal growth factor (all from Sigma Chemical Co., St. Louis, MO), and 5% horse serum (Life Technologies, Inc.). NIH3T3 cells were cultured in DMEM medium plus 10% calf serum. The exponential doubling times and saturation densities of the various cell lines were determined essentially as described previously (28). Cells were plated at a density of 1×10^4 cells per 35-mm-diameter well, in triplicate. Every 2 days, the cultures were refed with fresh medium, and the number of cells per well was determined using a Coulter counter. The doubling times were calculated from the initial exponential phase of the growth curves and the saturation densities from the plateau of the growth curves. Growth in soft agar was also performed as described previously (28). For the bottom layer of agar, 1 ml of 0.5% agar was placed in each 35-mm well of six-well plates. Then, 2 ml of 0.3% agar containing 1×10^5 cells were layered on top of the solidified layer of bottom agar. Colony formation was monitored by microscopy for up to 14 days, and the final numbers of colonies larger than 0.1 mm in diameter were determined. Plating efficiency assays were performed by seeding 1000 cells per 10-cm dish in complete medium. Cells were refed with fresh medium every 3–4 days for about 2 weeks. The cells were then fixed and stained with Giemsa, and the number of grossly visible colonies was counted.

All assays were performed in triplicate, and all experiments were repeated at least twice for all of the above-reported derivatives. The data reported in Table 1 are the results of a typical experiment for a representative cell line.

Construction of Retrovirus Vectors and Viral Transduction. The construction of the cyclin E retroviral expression plasmid PMV12-cycE and the method used for retrovirus packaging and transduction have been described previously (28). Briefly, to prepare infectious retrovirus particles, the PMV12-cycE plasmid or the control vector PMV12pl were transfected into the Ψ2 ecotropic (46) or the ΨAM amphotropic retrovirus packaging cell lines. The transfected cells were selected by growth in hygromycin (Boehringer Mannheim, Indianapolis, IN), and the cell-free media containing defective recombinant viruses were harvested, filtered, and used for the infection. Following selection for cells resistant to hygromycin (*hph*), several pools of thousands of resistant colonies were obtained and used for further analysis.

Characterization of Anchorage-independent Growth. G_0-synchronized cultures of NIH3T3 and Rat1 fibroblasts were prepared by incubation of subconfluent cultures in serum-free medium (DMEM supplemented with 5 μg/ml transferrin, 10 μg/ml bovine insulin, and 5 mg/ml BSA) for 48 or 72 h, respectively. Control studies showed that cells were >95% viable after this period, as assessed by trypan blue exclusion. Parallel adherent and nonadherent cultures were prepared according to a protocol described previously (32). Briefly, G_0-synchronized monolayers were trypsinized and replated into untreated (adherent cultures) or agar-coated (nonadherent cultures) tissue culture plastic dishes (6 ml of serum-free medium containing 1% agar in 10-cm dishes) in serum-free medium. After 6–10 h incubation (to allow cells in adherent cultures to adhere to the plate), calf serum was added to both adherent and nonadherent cultures (final concentration, 10%). Cells were collected at the indicated times by centrifugation and processed for flow cytometry or immunoblotting analyses. Alternatively, asynchronous, adherent cultures were trypsinized and replated into untreated or agar-coated dishes in medium containing 10% serum to obtain parallel adherent (monolayer) and nonadherent (suspension) cultures. For growth curves, cells were plated into untreated or agar-coated 35-mm dishes in complete medium, and 2 ml of fresh medium were added to the cultures every 2 days.

Flow Cytometric Analysis. Cells from parallel adherent and nonadherent cultures were collected by centrifugation, either directly (suspended cultures) or after trypsinization (adherent cultures) and washed twice with PBS. Cell pellets were resuspended in 1 ml PBS and fixed in 5 ml of 70% ethanol and stored at 4°C. For the analysis, cells were collected by centrifugation, and the pellets were resuspended in 0.2 mg/ml of propidium iodide in HBSS containing 0.6% NP40. RNase (1 mg/ml; Boehringer Mannheim) was added, and the suspension was incubated in the dark at room temperature for 30 min. The cell suspension was then filtered through a 41 μm Spectra mesh filter (Spectrum, Houston, TX) and analyzed for DNA content on a Coulter EPICS 753 flow cytometer. The percentage of cells in different phases of the cell cycle was determined using a ModFit 5.2 computer program.

DNA Synthesis. Cells were plated in triplicate in 24-well plates (10^4 cells/well) in the appropriate medium and grown for 24–36 h. The media were then changed, and the cells were incubated in the absence or presence of TGF-β1 (R&D Systems, Inc., Minneapolis, MN). At the indicated time points, the cultures were labeled for 1 h with [^{3}H]thymidine (1 μCi/ml; (Amersham Corp., Arlington Heights, IL) and then washed with ice-cold PBS and extracted with 10% cold trichloroacetic acid for 15 min on ice. After solubilization in 0.5 N NaOH, trichloroacetic acid-insoluble radioactivity was determined by liquid scintillation counting. Five ng/ml TGF-β1 in regular medium were used for the epithelial cell lines, whereas 10 ng/ml and 2 ng/ml in DMEM with 2% calf serum were used for Rat1 and NIH3T3 fibroblasts, respectively. Values are expressed as the percentage of [^{3}H]thymidine incorporation into the acid-insoluble fraction in the presence of TGF-β1 *versus* control values in the absence of TGF-β1. Qualitatively similar results were obtained after 24 and 72 h (data not shown).

Immunoreagents. The polyclonal antibody to cyclin E was obtained from Upstate Biotechnology (Lake Placid, NY). The monoclonal antibodies to cyclin E were purchased from PharMingen (San Diego, CA). The polyclonal antibody to $p27^{Kip1}$ was from Santa Cruz Biotechnology (Santa Cruz, CA).

Immune Complex Kinase Assay and Immunoblotting. Cdk enzyme assays were performed as described previously (28). Cell pellets were resuspended in kinase-lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 10 mg/ml leupeptin, and 10 mg/ml aprotinin (all of these chemicals were from Sigma)] and sonicated twice using a Sonifier cell disruptor (Ultrasonics, Inc., Plainview, NY). After centrifugation, clarified materials (50 mg) were incubated with protein A-Sepha-

rose for 1 h at 4°C for preclearing in IP buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, and 0.1% Tween 20]. Immunoprecipitations were carried out with 2 μg of the indicated antibody, and immunocomplexes were recovered with protein A-Sepharose. For H1 kinase activity, the protein A beads were washed four times with IP buffer, twice with washing buffer [50 mM HEPES (pH 7.5) and 1 mM DTT], and once with kinase buffer [50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate]. The final pellet was resuspended in 50 ml of kinase buffer supplemented with 2 μg of histone H1 (Boehringer Mannheim) and 5 μCi of [γ-^{32}P]ATP (Amersham) and incubated for 15 min at 30°C. The reaction was stopped by the addition of 25 μl of 2×-concentrated Laemmli sample buffer. The samples were separated on a SDS-10% polyacrylamide gel, and the phosphorylated histone H1 was visualized by autoradiography. Proteins from total cell lysates (50 μg) were used for Western blot analysis as described previously (28). Samples were electrophoresed by SDS-PAGE and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were then incubated with blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% Triton X-100, and 3% BSA) for 90 min at room temperature. Immunodetection was performed using the enhanced chemiluminescence (ECL) kit for Western blotting detection (Amersham).

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